The *embAB* genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol.

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Communicated by Arnold Demain, Massachusetts Institute of Technology, Cambridge, MA, August 5, 1996 (received for review April 30, 1996)

**abstract** The antimycobacterial compound ethambutol [Emb; dextro-2,2′-(ethylenedimino)-di-1-butanol] is used to treat tuberculosis as well as disseminated infections caused by *Mycobacterium avium*. The critical target for Emb lies in the pathway for the biosynthesis of cell wall arabinogalactan, but the molecular mechanisms for drug action and resistance are unknown. The cellular target for Emb was sought using drug resistance, via target overexpression by a plasmid vector, as a selection tool. This strategy led to the cloning of the *M. avium* *emb* region which rendered the otherwise susceptible *Mycobacterium smegmatis* host resistant to Emb. This region contains three complete open reading frames (ORFs), *embR*, *embA*, and *embB*. The translationally coupled *embA* and *embB* genes are necessary and sufficient for an Emb-resistant phenotype which depends on gene copy number, and their putative novel membrane proteins are homologous to each other. The predicted protein encoded by *embR*, which is related to known transcriptional activators from *Streptomyces*, is expendable for the phenotypic expression of Emb resistance, but an intact divergent promoter region between *embR* and *embAB* is required. An Emb-sensitive cell-free assay for arabinan biosynthesis shows that overexpression of *embAB* is associated with high-level Emb-resistant arabinosyl transferase activity, and that *embR* appears to modulate the in vitro level of this activity. These data suggest that *embAB* encode the drug target of Emb, the arabinosyl transferase responsible for the polymerization of arabinose into the arabinan of arabinogalactan, and that overproduction of this Emb-sensitive target leads to Emb resistance.

Ethambutol [Emb; dextro-2,2′-(ethylenedimino)-di-1-butanol] is a synthetic compound that has been known for its antimycobacterial activity since it was initially described in 1961 (1). It is one of the first-line drugs recommended for the treatment of disease caused by *Mycobacterium tuberculosis* (2) as well as opportunistic infections of AIDS patients caused by the *Mycobacterium avium* complex (3), and so it has broader application than isoniazid, the other widely used mycobacteria-specific drug which is effective only against *M. tuberculosis*. The genetic basis for Emb resistance has not been determined, and definition of the cellular target(s) has been complicated by the wide variety of disparate cellular processes that are disrupted by this drug (reviewed in ref. 4). However, the synergy against *M. avium* that is achieved when Emb is used in combination with drugs such as rifampicin (discussed in ref. 5) is most readily explained if the site of action of Emb is in arabinogalactan (AG) biosynthesis: disruption of the biosynthesis of AG would destroy the macromolecular assembly of the mycolyl-AG-peptidoglycan complex of the cell wall (6, 7), permitting drugs with intracellular targets (such as rifampicin) to enter the cell more easily.

Recent studies support the early work of Takayama and Kilburn (8) in defining the biosynthesis of the arabinan of AG as the critical target for Emb. It has been shown that Emb specifically inhibits the polymerization of cell wall arabinan (9), and that treatment of Emb-susceptible (Emb*) *Mycobacterium smegmatis* cells with Emb leads to the accumulation of β-D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA, or decaprenol phosphoarabinose) (10), a likely intermediate in arabinan biosynthesis. These findings led to the development of a cell-free assay to show that DPA is an arabinosyl donor for arabinan biosynthesis and that Emb inhibits arabinosyl transfer (11). The results also suggest that multiple arabinosyl transferase targets with varying sensitivities to Emb are present in mycobacteria (9, 11) and that these targets reside in the pathways for the biosynthesis of both AG and lipoarabinomannan (9). However, the biogenesis of AG appears to be more important for the antimycobacterial activity of Emb since a subinhibitory concentration of Emb still inhibits lipoarabinomannan biosynthesis, but not AG biosynthesis, in an Emb-resistant (Emb*) mutant of *M. smegmatis* (9).

In this report, we describe the cloning and sequencing of the *emb* region from *M. avium* that confers resistance to Emb when it is expressed in *M. smegmatis* on a multicopy vector. Genetic and biochemical evidence are presented to support our conclusion that the *embA* and *embB* genes encode for the arabinosyl transferase that is the primary cellular target for Emb.

**materials and methods**

**Bacterial Strains and Growth Conditions.** *M. smegmatis* strain mc²155 (12) and its transformants were grown in 7H11 (Difco) broth and agar medium unless otherwise noted. *Escherichia coli* strain χ2764 (13) or strains SURE, XL1-Blue or XL2-Blue (all from Stratagene) and their transformants were grown in Lennox L broth (GIBCO/BRL) and agar medium. All strains were incubated at 37°C except χ2764 which was grown at 30°C. Antibiotics (Sigma) were added to media at the following concentrations: kanamycin (Kan) at 10 μg/ml, tetracycline at 12.5 μg/ml, chloramphenicol at 100 μg/ml, and ampicillin at 100 μg/ml.

**Cloning Procedures.** A genomic library (14) of *M. avium* serovar 2, strain 2151, constructed by cloning 35- to 40-kb

Abbreviations: AG, arabinogalactan; DPA, decaprenol phosphoarabinose, or β-D-arabinofuranosyl-1-monophosphoryldecaprenol; Emb, ethambutol; Emb*, Emb-resistant; EmbB, Emb-susceptible; Kan, kanamycin; MIC, minimal inhibitory concentration.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U66560).

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partial Sau3A1 fragments of chromosomal DNA into the shuttle cosmid pYUB18 (15), was the source of cosmids pAEB1–pAEB22. Cosmid pAEB45, which was the target for Tn5 mutagenesis by standard procedures (16) using ATn5 (provided by Linda Lee, University of Texas Health Sciences Center, San Antonio), was constructed by trimolecular ligation of the 34-kb HpaI–DraI insert fragment from pAEB4, the 402-bp HincII fragment from pYUB18 (15) that contains the lambda cos site, and BsrI1107-linearized shuttle plasmid pYUB56 (17) (pYUB18 and pYUB56 were provided by William R. Jacobs, Jr., The Albert Einstein College of Medicine, Yeshiva University). The ligation reaction was packaged with the Gigapack Plus Kit (Stratagene) and the resulting phage particles were transduced into E. coli Y2764.

Subclones pAEB109–pAEB148 were generated by partial digestion of pAEB1 with Sau3A1 and ligation of the 10- to 12-kb fragments into the BamHI site of shuttle plasmid pMD31 (18), while subclones pAEB202 and pAEB203 were constructed by cloning the 8.3-kb SstI fragment from pAEB148 in both orientations into the SstI site of the mycobacterial integrating vector pMH94 (19) (these vectors were provided by Graham F. Hatfull, University of Pittsburgh). Plasmids pWRH8 and pWRH10 were produced by treating the 8.3-kb SstI fragment from pAEB1 with mung bean nuclease (Stratagene) prior to ligation with the 6.4-kb PvuI11 fragment of pMD31 (referred to as pMD31APvu) in both orientations. Deletion derivatives were constructed by digesting pWRH8 and pWRH10 with SphI and religating the purified 14-kb fragments. E. coli transformations were carried out by the one-step procedure (20), electroporation (Bio-Rad), or by using commercially-available competent cells (strains SURE or XL2-Blue from Stratagene). Plasmid DNA was isolated from E. coli by alkaline lysis (20) or Qiagen (Chatsworth, CA) columns, and characterized by restriction analysis prior to transformation of M. smegmatis by electroporation (15). Plasmids were recovered from M. smegmatis transformants by electroelution into E. coli (21), or by isolating plasmid DNA via alkaline lysis (20) of cells disrupted with 0.5 mm zirconium beads followed by transformation of E. coli.

**Drug Resistance Assays.** M. smegmatis mc2155 transformants were plated in duplicate on medium containing Kan with or without Emb at 0.75 μg/ml. Isolates were scored as Emb if growth appeared on both types of plates after a 3-day incubation. The minimal inhibitory concentration (MIC), determined by plating on medium containing Kan plus 0–2.5 μg/ml of Emb in increments of 0.25 μg/ml, was defined as the first concentration of Emb that inhibited 99–100% of growth after 3–5 days of incubation. Control strains contained the appropriate cloning vector.

Disk susceptibility assays were performed with mc2155 transformed with either pAEB1 or the cloning vector pYUB18. Overlays consisting of 200 μl of a saturated culture and 5 ml of soft agar were poured onto 100-mm plates of agar medium and allowed to harden. Paper disks containing the following drugs or antibiotics were fixed to the agar surface: 25 μg of capreomycin, 10 μg of Emb, 25 μg of ethionamide, 15 μg of isoniazid, 5 μg of rifampin, and 5 μg of streptomycin. The zones of inhibition were examined after 3 days of incubation.

**DNA Hybridization.** Colony blot hybridizations of E. coli transformants were carried out on nitrocellulose disks by standard procedures (20). For Southern blot analysis (20), ~2 μg of mycobacterial chromosomal DNA was digested with EcoRI. DNA from Mycobacterium leprae and M. tuberculosis were obtained from National Institutes of Health Contracts AI52562 and AI25147, respectively, at Colorado State University, while the others were isolated by the modified bead beater method (14). Restriction fragments were nonradioactively labeled with digoxigenin using the Genius Kit (Boehringer Mannheim). Hybridization and washes were performed under stringent conditions, and the results were visualized by colorimetric detection (for colony blots) or by exposure to x-ray film following incubation with Lumi-Phos 530 (for Southern blots), according to the manufacturer’s instructions (Boehringer Mannheim).

**DNA Sequencing.** Overlapping restriction fragments that spanned the Emb resistance region were subcloned from pAEB1 into pBlueScript SK+ (Stratagene), and then nested deletions were generated using the Exo/Mung DNA Sequencing System (Stratagene). Double-stranded DNA templates were sequenced using the Sequenase version 2.0 Kit (United States Biochemical) with pBlueScript reverse and ~20 primers (Stratagene). Custom primers were synthesized (Macromolecular Resources Facility, Colorado State University) as necessary to resolve sequence ambiguities and possible frameshift errors. Contiguous DNA sequences were constructed with the Sequence Assembly Manager (Molecular Biology Information Resource Program, Baylor College of Medicine) and potential frameshifts in the sequence were identified with Large Scale Sequence Analysis Suite (Keith Robison, Harvard University; made available to us by Douglas Smith). The DNA sequence was analyzed by the Staden (22), PGGENE (IntelliGenetics), and Genetics Computer Group (GCC, version 10.1) sequence analysis programs using default parameters. ORFs were identified with codon usage tables derived from the sequence of mycobacteriophage L5 (23). Predicted amino acid sequences were analyzed with FASTA against the Protein Identification Resource, SwissProt, and Genpept data bases.

**Protein Analysis.** M. smegmatis containing either pMD31 or pAEB148 was grown in glycerol-alanine-salts broth containing Kan or Kan plus 0.75 μg/ml Emb, respectively. Approximately 5 g of stationary phase cells was disrupted in a Bead Beater (Biospec, Bartlesville, OK) with 0.1 mm zirconium beads using 10 cycles of 10-sec pulses and 30-sec cooling periods. Following centrifugation at 500 × g to remove beads and any unbroken cells, the supernatant was centrifuged at 28,000 × g for 30 min. Protein concentrations of the resuspended pellet, containing cell wall and membrane proteins, and the supernatant, containing membrane and cytosolic proteins, were determined using the BCA assay (Pierce). Equivalent amounts of each sample (5 μg) were analyzed by SDS/10% PAGE.

**Whole-Cell Radiolabeling Experiments.** Duplicate cultures of mc2155 containing pMD31 or pAEB148 were grown to an optical density of 0.6 at 600 nm in glycerol-alanine-salts broth containing 0.05% Tween and Kan. Emb was then added to one set of flasks at a concentration of 1 μg/ml while all flasks received 1 μCi/ml of L-[14-C]U-glucose (ICN, 250 μCi/mM; 1 Ci = 37 GBq). After 4 more hours of incubation, the cells were harvested and washed with buffer. The lipolysarobinoman-fucycol-AG-peptidoglycan complex, prepared by ethanol reflux of delipidated cells as described (9), was hydrolyzed for 2 h at 120°C with 2 M CF3COOH prior to extraction with CHCl3 to remove fatty acids. The monosaccharides released by acid treatment were analyzed by HPLC (Dionex, Sunnyvale, CA) using a CarboPack PA1 anion-exchange column (4 × 250 mm) and isocratic elution at 1 ml/min with 100 mM NaOH. The stream was split between a pulsed amperometric detector and a β-RAM detector (Insus Systems, Tampa, FL). A mixture of unlabeled standards (arabinose, galactose, glucose, mannose, rhamnose, and ribose) was cojected with a separate sample to determine peak identities.

**Cell-Free Arabinosyl Transferase Assays.** The incorporation of [14C]-α-arabinose from DPA into arabamin was assayed as described previously (11). Briefly, cell sonicates prepared from cultures grown in glycerol-alanine-salts broth were centrifuged at 27,000 × g, and the supernatant was recentrifuged at 100,000 × g to yield the membrane pellets containing the enzyme activity (0.4–0.5 mg of protein per reaction). The acceptors for the reaction, provided by the particulate cell wall fraction (0.2–0.3 mg of protein per reaction), were prepared by
fractionating the 27,000 × g pellet on a 60% Percoll gradient, while the radiolabeled arabinose donor (20,000 cpn, 9 μM per reaction) was chemically synthesized. The products of the reaction were separated by descending paper chromatography, and the radioactivity retained at the origin was counted to determine incorporation (typically 30% in the absence of Emb) into polymer.

RESULTS

Cloning and Characterization of the Emb-Resistance Region. The cellular target for Emb was sought using drug resistance, via target overexpression by a plasmid vector, as a selection tool. A genomic library of DNA from a moderately Embr (MIC of 14 μg/ml) strain of M. avium was screened in the Embr (MIC of 0.25 μg/ml), electroportable host, M. smegmatis strain mc²155, for clones that produced an Emb phenotype. Direct selection of transformants on medium containing Kan and 2.5 μg/ml of Emb led to the identification of six clones that conferred a 10-fold increase in Emb MIC. Comparison of two overlapping clones, pAEBr and pAEBr4 (Fig. 1A), defined a resistance region of 22.5 kb. Disk susceptibility assays with other antimycobacterial drugs (including capreomycin, ethionamide, isoniazid, rifampin, and streptomycin) indicated that resistance was specific for Emb (data not shown).

The region required for Emb resistance was further examined by transposon mutagenesis of pAEBr4, a Kan-susceptible derivative of pAEBr4. The resulting composite map of the Tn⁵ insertion sites that localized the Emb resistance region to within 9.8 kb, based on flanking insertions which did not affect drug resistance, is shown in Fig. 1B. At the same time, the region required for Emb resistance was also defined by overlapping clone analysis. Cosmids from the genomic library were identified by colony blot hybridization using a probe constructed from the internal 7.1-kb EcoRI fragment common to pAEBr1 and pAEBr4 (Fig. 1A) and then tested for Emb resistance in M. smegmatis. The region needed to produce Embr transformants was narrowed down to the 10.3 kb of DNA shared by clones pAEBr21 and pAEBr22 (Fig. 1A); however, these cosmids conferred only about half the Emb MIC (1.25 and 1.0 μg/ml, respectively) obtained with the original clones. The resistance regions identified by Tn⁵ mutagenesis and overlapping clone analysis thus coincided, but the latter results suggested that flanking sequences might affect the level of Emb resistance. This possibility was addressed by screening a 10–12-kb sublibrary of pAEBr1, and Embr clones pAEBr38 and pAEBr109 defined a smaller essential region of 7 kb (Fig. 1C). Since all the Embr subclones had a resistance level of 2.5 μg/ml of Emb, the same as that obtained with pAEBr1, it was concluded that the lower MICs obtained with overlapping clones pAEBr21 and pAEBr22 were possibly due to the instability of those particular inserts in M. smegmatis.

The Emb-Resistance Region Is Common to Other Mycobacteria. The ubiquity of the Emb-resistance region was investigated by Southern blot analysis with genomic DNA from M. avium, M. smegmatis, M. tuberculosis, and M. leprae. Using the 7.1-kb EcoRI fragment (Fig. 1) as a probe, hybridizing bands were detected in all of the mycobacterial species tested, although there were variations in signal intensity (Fig. 2). These results indicated that the Emb-resistance region (emb) was not unique to Embr mycobacteria and thus did not define an Emb-resistance determinant per se. This conclusion was supported by the cloning of emb homologs from a number of Embr or Emb strains, including M. smegmatis mc²155, M. avium serovar 2 strain 724, and M. tuberculosis strains H37Rv and CSU24 (data not shown).

Sequence of the emb Region. The sequence of the 9543-bp EcoRI–EcoRV fragment encompassing emb from M. avium strain 2151 was determined. The G+C content of 70.4% was consistent with mycobacterial DNA (24), and the region contained three complete ORFs, designated embR, embA, and embB (indicated in Fig. 1B). The GTG start codon for embR was chosen by its proximity to a potential AGGAGG ribosome-binding site with homology to the 3'–OH end of 16S ribosomal RNA from M. leprae (25). The product of embR is predicted to be a 384-aa protein with a calculated Mr of 41,267.

![Fig. 1. Genetic map of the M. avium Emb-resistance region. (A) Restriction maps of the inserts from representative cosmids identified by direct selection for Emb resistance or by overlapping clone analysis. (B) Transposon Tn5 insertion map relative to a restriction map and the predicted ORFs (the direction of transcription of embR, embA, and embB is denoted by arrows). Tn5 inserts which abolish (•) or have no effect (○) on Emb resistance are indicated. (C) Restriction maps of the inserts of representative SacI3A1 subclones of pAEBr1. Resistance levels of the clones are given on the right: +, MIC >1.0 μg/ml Emb; −, no resistance at 0.25 μg/ml Emb; B, BglII; E, EcoRI; V, EcoRV; K, KpnI; S, Scal; T, SstI; c, vector Clal; d, vector DraI; k, vector KpnI; x, vector XbaI.](image)

![Fig. 2. Agarose gel of EcoRI-digested mycobacterial chromosomal DNAs and corresponding Southern blot using the 7.1-kb EcoRI fragment from pAEBr1 as the probe. Lanes: 1 and 6, pAEBr1; 2, M. avium serovar 2, 2151; 3, M. smegmatis mc²155; 4, M. tuberculosis H37Rv; 5, M. leprae; 7, 1-kb DNA marker (GIBCO/BRL).](image)
It shows good similarity over its entire length with members of a family of transcriptional activators in Streptomyces, including RedR (26), ActII-orf4 (27), the N-terminal domain of ArsR (28), and Dnr I (29); the highest score obtained by FASTA analysis was with the S. peucetius Dnr I protein, with 33.5% identity over a 254-aa region (data not shown). The embR coding region is separated from embA and embB by a 178-bp divergent promoter region. The GTG start codon of embA was assigned by its proximity to a potential ribosome-binding site, GAG, while embB lacks a potential ribosome-binding site and thus appears to be translationally coupled to embA: the TG of the TGA stop codon of embA is shared by the proposed ATG start codon of embB (data not shown). The respective deduced amino acid sequences of embA and embB, 1108 aa (MW = 117,372) and 1065 aa (MW = 114,642), show no similarity to other proteins in the database but they are similar to each other, with 44.8% identity (63.2% similarity) along their entire lengths (Fig. 3). The products of embA and embB are predicted to have 8 and 10 potential membrane-spanning domains, respectively (see Fig. 3).

The Emb' Phenotype Requires Only embA and embB. Alignment of the ORFs with the maps of the subclones suggested that only embA and embB were required for Emb resistance. Comparison of the inserts of pAEB138 and pAEB121 (Fig. 1C), together with the locations of Tn5 insertions in embB that resulted in Emb-susceptibility (Fig. 1B), clearly indicated that embB was essential. Similarly, embA was shown to be dispensable by comparison of pAEB148 and pAEB109, while the Emb-susceptibility of pAEB144 indicated that an intact divergent promoter region was required. Since transposon insertions in embA could have polar effects on the expression of embB, an in-frame deletion was constructed to determine if embA was needed for resistance. The 8.3-kb SsrI fragment (shown on the map of pAEB148 in Fig. 1C) was cloned from pAEB1 into pMD31APvu in both orientations to produce pWHR8 and pWHR10, both of which confer MICs of 2.5 

μg/ml of Emb. Deletion of the 660-bp SphI fragment, which removed 220 internal amino acids (aa positions 544–763 in Fig. 3) but maintained the reading frame of embA, resulted in the loss of Emb resistance. Together these results indicated that both embA and embB are required for the Emb' phenotype.

Copy Number Determines the Level of Emb Resistance. The Emb resistance level of 10-fold over background correlated with the predicted copy number of 5 to 10 for the cloning vectors (15), and a strong linear relationship was noted between the concentration of Emb used for selection of mc2155 transformants and the number of Emb' colonies obtained (data not shown). To see if resistance was influenced by copy number, embAB was inserted into the nonreplicating, integrative vector pMH94 which contains attP and int from mycobacteriophage L5 (19). When both orientations of the 8.3-kb SsrI fragment were cloned from pAEB148 into the SsrI site of the vector (to produce pAEB202 and pAEB203), and the constructs were integrated into the attB site of the M. smegmatis chromosome (data not shown), the MIC dropped to 2-fold over background. Since the same SsrI fragment produced a 10-fold increase in Emb resistance when it was cloned in a replicating plasmid (pWHR8 and pWHR10, above), these results indicated that the level of Emb resistance mediated by the emb region was not an intrinsic property of the cloned DNA but depended on gene copy number.

EmbA and EmbB Are Overexpressed in M. smegmatis. The above observation that gene copy number determined the Emb resistance level suggested that the embAB gene products might be overexpressed in M. smegmatis. Crude pellet and supernatant fractions of mc2155 containing either pAEB148 or the cloning vector pMD31 were compared by polyacrylamide gel electrophoresis. As shown in Fig. 4, the cell wall–membrane fraction from cells harboring pAEB148 contains a significant increase in a band of protein(s) above the 97.4-kDa marker, consistent in size with those predicted for the EmbA and EmbB proteins (117- and 115-kDa, respectively), while this increase was not observed in the vector control.

Cell Wall Arabinan Biosynthesis Proceeds Normally in Emb-Treated Cells Containing embAB. It was shown in previous studies that the treatment of 14C-glucose-labeled M. smegmatis mc2155 cells with Emb severely diminished the incorporation of radiolabeled arabinose into AG (9). Using similar techniques, the effect of Emb was examined using strains of mc2155 transformed with either pMD31, the cloning

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**Fig. 3.** Alignment of the embA and embB gene products using the BESTFIT program (Genetics Computer Group). , Identical amino acids; ( ), conserved amino acids; ( ), semiconserved amino acids. The putative membrane-spanning domains identified by the program RAOARGOS (IntelliGenetics) are underlined.

**Fig. 4.** Protein profiles of the cell wall–membrane fractions of M. smegmatis mc2155 containing the cloning vector pMD31 (lane 1) or pAEB148 (lane 2) relative to size standards (lane 3; Gibco/BRL) visualized by silver staining of a 10% polyacrylamide gel. The overexpressed protein band (lane 2) is denoted by an arrow.
vector, or pAEB148, a pMD31-derived plasmid containing the embAB genes. Emb treatment caused a dramatic decrease in the amount of $^{14}$C-labeled arabinose in AG when pMD31 was present in M. smegmatis, while Emb did not have an effect when the host cells contained pAEB148 (Fig. 5).

Association of embAB with Emb$^r$ Arabinosyl Transferase Activity and Effect of embR on the Level of Emb Resistance Achieved in a Cell-Free Assay. It was previously shown that Emb inhibits the incorporation of radiolabeled arabinose from the lipid carrier, DPA, into a polymer of arabinan (11). This same cell-free assay was used to determine if the above effects of Emb on AG biosynthesis in whole cells could be directly related to arabinosyl transferase activity in vitro. Maximal inhibition of arabinosyl transferase activity was determined from dose-response curves with increasing concentrations of Emb, and three types of Emb resistance were observed. Cell-free preparations of M. smegmatis mc$^2$155 with or without the cloning vector pMD31 were the most sensitive to Emb, with only 26–29% residual arabinosyl transferase activity in the presence of 50 mg/ml Emb (representative results are shown in Fig. 6); these results are consistent with previous studies that indicated that this assay supports a number of arabinosyl transferases and that one or more are intrinsically resistant to Emb (11). In contrast, preparations from cells containing pAEB1 or pAEB148 were highly resistant to Emb, with 63–65% of the arabinosyl transferase activity remaining Emb$^r$, while that obtained with pAEB109, as well as an Emb$^r$ mutant of mc$^2$155 (9), displayed intermediate levels (37–40%) of Emb$^r$ arabinan biosynthesis (representative results are shown in Fig. 6). As was noted earlier, the major difference between pAEB148 and pAEB109, which both contain embAB, is the presence or absence of embR, respectively (see Fig. 1C).

A priori, it might be expected that the amount of the Emb-sensitive target present in each cell-free preparation would dictate the concentration of Emb required for inhibition, and that all extracts would eventually achieve the same saturated background activities, assuming that the amount of arabinose acceptors remains constant. Since the initial levels of endogenous arabinose acceptors are about the same for all the preparations tested, as judged by the similar amounts of radiolabel that each can incorporate in the absence of Emb (see Materials and Methods), the observed differences in maximum levels of inhibition likely reflect the differential abilities of the cell-free extracts to support the synthesis of intermediate arabinose acceptors, as well as final arabinan products, in the presence of Emb.

**DISCUSSION**

These studies suggest that the embAB genes of M. avium encode for the primary cellular target for Emb, namely the arabinosyl transferase III responsible for the polymerization of arabinose into the arabinan of AG (9). This conclusion was supported by the ability of the embAB genes to specifically confer an Emb-resistant phenotype that depends on gene copy number, consistent with drug resistance resulting from overexpression of the drug target. In addition, overexpression of these genes permits normal incorporation of arabinose into AG in the presence of Emb. Although these results could not rule out the possibility that the observed Emb resistance was due to a novel mechanism for drug exclusion or efflux, the cell-free assays for arabinan biosynthesis clearly showed that embAB are associated with high-level Emb$^r$ arabinosyl transferase activity. These genes are ubiquitous amongst mycobacteria regardless of the strains' Emb susceptibility levels, as would be expected for genes involved in cell wall biosynthesis. Since recent studies indicate that multiple-drug resistance in M. avium and M. tuberculosis usually results from the accumulation of mutations in genes encoding the drug targets (reviewed in ref. 30), it can be predicted that Emb$^r$ clinical isolates will have up-regulatory mutations in embR or the divergent promoter region, or have altered embA or embB structural genes.

A working hypothesis that takes into account the potential translational coupling of embAB, the mechanism of action of EmbA and EmbB, and the possible role of EmbR, has been formed by using secondary metabolite biosynthesis as a model.
Although secondary metabolites vary in composition and function, the synthesis of these complex molecules always occurs from the condensation of single identical starter units. Mycobacterial arabinan may be considered similar in this regard since this elaborate homopolyasaccharide is generated from multiple D-arabinofuranose units (7). The translational coupling that is predicted for embAB is usually associated with genes whose protein products are required in equimolar amounts, such as those functioning in multienzyme complexes (31), and this feature, together with the amino acid sequence similarity of EmbA and EmbB, is found among enzymes involved in secondary metabolite biosynthesis. For example, the antibiotics actinorhodin and gramicidin are synthesized from heterodimeric enzyme complexes which are composed of two translationally coupled proteins sharing 49% aa similarity (32, 33). Likewise, the erythromycin gene cluster encodes for three ORFs, two of which are translationally coupled, with 64% or higher amino acid similarity (34). The target of Emb may thus be a heterodimeric enzyme complex derived from EmbA and EmbB. The identification of embR, with its high degree of similarity to transcriptional activators which regulate secondary metabolite synthesis, is compatible with this hypothesis.

A role for embR in modulating expression of embA and embB was originally suggested by the characteristic genetic organization of regulatory protein-divergent promoter region-structural gene (35), and this model is supported by the decrease in the level of Embα arabinosyl transferase activity observed when embR is missing. EmbR lacks a helix-turn-helix motif but is similar (27.1% identity over a 129 aa region; data not shown) to ToxR, a transcriptional activator for choler toxin production (36). The region of similarity encompasses a 96-aa domain of the N terminus of ToxR that associates with directly repeated sequences in the promoter region of the cto operon (36), and the divergent promoter region between embR and embA embB contains two direct DNA repeats of CGGCGCGGA and one related sequence (CGGCGCGGA) (data not shown). Curiously, embR does not appear to influence the phenotypic expression of Emb resistance by whole cells, although the intact divergent promoter region is required. It is possible that the host cells, which presumably contain an embR homolog, provide sufficient complementation in trans to produce an Embα phenotype that is not distinguishable from that of embR-containing cells until examined at the cellular level. Future studies should resolve these questions and provide further insights into the regulation of mycobacterial cell wall biosynthesis.

We thank Douglas Smith and Graham Hatfull for assistance with DNA sequence analysis; Richard Lee for the synthesis of radiolabeled DPA; Michael McNeil for assistance with the Dionex HPLC; Michael Sonnenberg for assistance with protein analysis; and William Howe and Caroline Morehouse for excellent technical assistance. This work was supported by Grants AI30189 and AI38087 of the National Cooperative Drug Discovery Group-OI, and AI38087 of the National Institute of Allergy and Infectious Diseases (P.J.B., Program Primary Investigator); Public Health Service Grants AI33706 (Michael R. McNeil, Primary Investigator) and AI01185 (to J.M.I.); the Colorado Agricultural Experiment Station, and Animal Health and Disease Formula funds (to J.M.I.).


